

Mutations in the *Drosophila melanogaster* gene *three rows* permit aspects of mitosis to continue in the absence of chromatid segregation

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SUMMARY

We have cloned the *three rows* (*thr*) gene, by a combination of chromosome microdissection and P element tagging. We describe phenotypes of embryos homozygous for mutations at the *thr* locus. Maternal mRNA and protein appear to be sufficient to allow 14 rounds of mitosis in embryos homozygous for *thr* mutations. However, a small percentage of cells in syncytial blastoderm stage *thr* embryos sink into the interior of the embryo as if they have failed to divide properly. Following cellularisation all cells complete mitosis 14 normally. All cells become delayed at mitosis 15 with their chromosomes remaining aligned on the spindle in a

metaphase-like configuration, even though both cyclins A and B have both been degraded. As cyclin B degradation occurs at the metaphase-anaphase transition, subsequent to the microtubule integrity checkpoint, the delay induced by mutations at the *thr* locus defines a later point in mitotic progression. Chromosomes in the cells of *thr* embryos do not undertake anaphase separation, but remain at the metaphase plate. Subsequently they decondense. A subset of nuclei go on to replicate their DNA but there is no further mitotic division.

Key words: metaphase delay, mitosis, *Drosophila*, *three rows*

INTRODUCTION

Thirteen cycles of rapidly alternating DNA synthesis and nuclear division follow the fertilisation of a *Drosophila melanogaster* egg. These cycles are unusual in at least two ways. Firstly, the syncytial division cycles lack the checkpoint that prevents entry into mitosis in the presence of unreplicated DNA. Thus injection of aphidicolin into embryos prevents DNA replication but has little effect on mitosis, with both centrosome replication and nuclear envelope breakdown cycles proceeding normally (Raff and Glover, 1988). Secondly, the mitotic A- and B-type cyclins do not undergo complete degradation at the metaphase-anaphase transition in the rapid division cycles (Maldonado-Codina and Glover, 1992).

After nuclear division 7 the majority of nuclei begin to migrate towards the periphery of the embryo, although some remain in the interior, cease dividing and become the polyploid yolk nuclei. The dividing nuclei have approached the surface by the time of the tenth nuclear division and form a monolayer just beneath it. During migration and in the syncytial blastoderm stages, when the nuclei are at the cortex, the time taken for each cell cycle increases, from 9 minutes for cycle 8 to 21 minutes for cycle 13 (Foe and Alberts, 1983). If any of divisions 11 to 13 at the embryo periphery are aberrant the affected nuclei may sink towards

the central part of the embryo (Sullivan et al., 1990). This loss of nuclei from the surface monolayer appears to be a generalised reaction to defective chromosome segregation (Minden et al., 1989; Warn et al., 1987). A small subset of nuclei become cellularised immediately upon reaching the posterior pole to form the pole cells, precursors of the adult germline. These cells no longer divide synchronously with the syncytial nuclei. Cellularisation of the remaining nuclei takes place in the interphase of cycle 14 following four further divisions at the cortex.

Maternally provided gene products are sufficient to drive the syncytial cycles (Szabad and Bryant, 1982). Mutations in these maternally expressed genes can give rise to mitotic defects in early embryos. Some of these genes, for example *giant nuclei* (*gnu*) (Freeman et al., 1986; Freeman and Glover, 1987), encode proteins that are apparently only required in the syncytial embryo. Females homozygous for mutations at the *gnu* locus produce eggs that undergo multiple rounds of DNA replication without any nuclear division. Centrosome replication occurs independently of the formation of these giant nuclei (Freeman et al., 1986; Freeman and Glover, 1987). Maternal effect mutations in other genes, *plutonium* and *pan gu*, have also been described to have similar phenotypes (Shamanski and Orr-Weaver, 1991). The autonomous replication of centrosomes appears to be a general consequence of disrupting mitosis in syn-

cytial embryos and may be seen, for example, with combinations of mutant alleles of *abnormal spindle (asp)* that show a maternal effect mitotic phenotype (González et al., 1990).

For many of the gene functions necessary for mitosis the maternally provided mRNAs and proteins last until the third larval instar (Gatti and Baker, 1989). Thus a zygote homozygous for a mutation in certain genes essential for mitosis can survive until this late stage using the wild-type product supplied by its heterozygous mother. This perdurance of maternal protein has been clearly demonstrated for the *asp* product (Carmena et al., 1991), and it is inferred from the zygotic phenotype of other homozygous mutants lacking protein phosphatase *PPI 87B* (Axton et al., 1990), or a myosin light chain, *spaghetti-squash* (Karess et al., 1991), amongst other examples.

Other maternally supplied gene products only last until the time of cellularisation or shortly thereafter. The pre-eminent example is the product of the gene *string (stg)*, a member of the *cdc25* family of mitotic inducers (Edgar and O'Farrell, 1989). Maternal transcripts of this gene become degraded at the time of cellularisation and this introduces an extended G₂ phase into the later cell division cycles. Groups of cells (mitotic domains) enter mitosis 14 in a characteristic pattern (Foe, 1989) anticipated by the spatially and temporally regulated zygotic transcription of *stg* some 20 to 25 minutes earlier. In contrast to the syncytial divisions, the mitotic cyclins A and B now show distinct degradation at the metaphase-anaphase transition in the cells of the mitotic domains (Whitfield et al., 1990; Lehner and O'Farrell, 1989, 1990). There appears to be persistence of sufficiently high levels of maternal *cyclin A* transcripts to permit mitoses 14 and 15 in the absence of zygotic expression. Embryos mutant for zygotic expression of *cyclin A* arrest in the interphase before mitosis 16 (Lehner and O'Farrell, 1990). Embryos in which both *cyclin A* and *cyclin B* gene activities have been removed arrest during the interphase preceding mitosis 15. This synthetic defect indicates some synergy of function between these two activities (Knoblich and Lehner, 1993).

While *stg* mutants are unable to enter mitosis 14 they are capable of considerable further development. However, the reduced number of cells means that although they differentiate segmental cuticular structures these are missing pattern elements (Hartenstein and Posakony, 1990). Work on the partial rescue of *stg* mutants, using a heat-inducible *stg* transgene, shows that there is a close correlation between the number of post-blastoderm mitoses completed and the number of rows of epidermal denticles that subsequently differentiate (Edgar and O'Farrell, 1990). The embryonic zygotic lethal mutation *three rows (thr)* was originally isolated because of its cuticular phenotype, a reduction in the number of rows of epidermal denticles (Nusslein-Volhard et al., 1984). Consideration of the arguments outlined above for *string* led to the idea that the cuticle phenotype in *thr* mutants could be explained by mitotic defects (Tearle and Nusslein-Volhard, 1988). In this paper we demonstrate that abnormal mitosis does indeed occur in *thr* embryos, and we characterise some aspects of the mitotic defects.

MATERIALS AND METHODS

Molecular cloning

In order to clone the *thr* gene, DNA from *thr*^{BH9.8}/CyO flies was digested with *Xba*I and ligated into Dash II (Stratagene), which was plated on the selective host *Escherichia coli* Q359 (Frischauf et al., 1983) to produce a library. DNA was obtained from polytene band 54F by microdissection of salivary gland chromosomes from wild-type *D. simulans* (a *D. melanogaster* sibling species) and amplified by PCR (Saunders et al., 1989). Microdissection was performed on *D. simulans* chromosomes in order to minimise problems associated with transposable elements within the dissected chromosome region (Sidén-Kiamos et al., 1990). The microcloned DNA hybridised exclusively to 54F-55A in situ. Plaques from the plated library were transferred onto duplicate filters for separate hybridisation with probes derived from the P element (p 25.1) and the PCR amplified microdissected DNA. Only clones hybridising to both probes were purified. These contain an insert comprising 18 kb of *Drosophila* DNA and a 2.9 kb P element. One such clone, 81.2 (Fig. 1), was used for further studies. A 5.2 kb *Eco*RI fragment from this phage was used to probe the Lorist 6 cosmid library of wild-type DNA, described by Sidén-Kiamos et al. (1990). Six distinct clones were obtained from this screen, one of which is 15C2 (Fig. 1), which contains some 35 kb of *Drosophila* DNA that hybridises in situ to 54F3-6 on chromosome arm 2R. Altogether 50,000 plasmid clones from a 0-4 h embryonic cDNA library (Brown and Kafatos, 1988) were screened with a mixture of the 3.4 kb *Hind*III fragment and the 1.4 kb and 1.05 kb *Eco*RI-*Sac*I restriction fragments from 15C2, which lie in the vicinity of the P element insertion site. One cDNA clone with an insert size of approximately 2.5 kb (2B1) and two smaller clones (2A1, 3A1) were recovered. All of these cDNA clones are incomplete, lacking sequence at the 5' end.

thr alleles

The EMS (ethylmethanesulphonate)-induced *thr*^{1B} allele and the P element-induced *thr*^{9.8} allele were used in the characterisation of the phenotype and the *thr*^{1B} and *thr*^{SJB22} alleles (both EMS-induced) were used in the rescue transformation experiments.

Germline transformation and rescue

This was performed essentially as described by Axton et al. (1990). Two constructs were separately microinjected into w; 2-3 *Sb e/TM2* embryos (Robertson et al., 1988). The larger, N15, is a 15 kb *Not*I fragment of cosmid 15C2 in the pCas4 vector; 10 transformed lines were recovered from 49 fertile G₀ adults (20% transformants). The smaller construct, RX2, is a 9 kb fragment excised from cosmid 15C2 with *Xba*I and *Eco*RI partial digestion and ligated into the pw8 vector (Klemenz et al., 1987); 8 transformed lines were recovered from 62 fertile G₀ adults (13% transformants). The ability of the RX2 construct to rescue the lethality of *thr* mutations was tested using a line in which the transforming transposon was carried on the X chromosome. Females from such a line (*w*^a P[*w*⁺, RX2]; *SM1 sp*/+) were crossed to *w*¹¹¹⁸/Y; *thr sp*/CyO males. The *w*⁺; *sp* F₁ progeny were crossed inter se. The presence of *w*⁺; *Cy*⁺ *sp*⁻ flies in the F₂ progeny indicates that the RX2 construct can rescue lethality of *thr*^{1B} mutations. A similar approach, but using *CyO dp* in place of *SM1 sp*, was used to show that RX2 will rescue flies homozygous for *thr*^{SJB22}.

Sequence analysis

cDNA 2B1 was cleaved into two *Eco*RI fragments of 1.7 and 1.1 kb and these were each ligated into both of the pBluescript® KS+ and pBluescript® SK+ vectors (Stratagene). These constructs were oriented so that the sequencing primer (M13-20) was closest to

the 5' ends of both fragments in pKS+ and to the 3' ends in pSK+. Series of nested exonuclease III deletion subclones were prepared, starting from the *Xba*I site of pKS+ (5' to 3') and from the *Xho*I site of the pSK+ (3' to 5'), by the method of Henikoff (1987). Double-stranded plasmid deletion subclones were packaged as single-stranded phage by superinfection with M13 MK07 helper phage and prepared for dideoxynucleotide sequencing by standard procedures (Sambrook et al., 1989); 0.5 and 1.7 kb *Sal*I/*Hind*III cDNA fragments were also sequenced. We were unable to isolate a full-length cDNA from available libraries and so determined the sequence of the 5' end of the gene from genomic DNA. The appropriate 2.4, 1.6 and 4 kb *Eco*RI genomic fragments were cloned into pKS+ and pSK+, and deletion series were constructed starting from the *Sac*II site of KS and the *Sal*I site of SK; 2.3 kb *Spe*I/*Eco*RI and 560 bp *Pst*I/*Hind*III fragments of genomic DNA were also sequenced. In order to positively identify the 5' end of the *thr* mRNA we used nested PCR primers, single-stranded ligation of an oligonucleotide anchor to single-stranded cDNA (SLIC) and rapid amplification of cDNA ends (RACE) PCR to obtain a cDNA fragment corresponding to this region of the message (Clontech 5' AmpliFINDER RACE kit). The resulting PCR product was sequenced using ³²P-end-labelled specific sequencing primers and the BRL dsDNA cycle sequencing system. Accumulated sequence was assembled using the Microgenie software.

Immunostaining of embryos

Embryos were collected, dechorionated and fixed in paraformaldehyde as described previously (Maldonado-Codina and Glover, 1992) with the following modifications. A balancer chromosome *CyO ftz-lacZ* (kind gift from Sarah Bray) was used to distinguish *thr*⁺ embryos from their homozygous mutant siblings. Taxol was only used when microtubule distribution was to be examined and instead of immediately devitellinising embryos following fixation they were washed overnight in 0.1% Triton X-100 in PBS (PBTrX). Embryos were then stained for β -galactosidase activity by incubation in 0.28% X-gal in Fe/NaP buffer (pH 7.2, 7.2 mM Na₂HPO₄, 2.8 mM NaH₂PO₄, 3.05 mM K₃Fe(CN)₆, 3.05 mM K₄Fe(CN)₆, 150 mM NaCl and 50 mM MgCl₂) and washed twice in PBTrX. Methanol devitellinisation, antibody staining and

mounting were as previously described (Whitfield et al., 1990). Images were captured using a Bio-Rad MRC 600 laser-scanning-confocal head, in conjunction with a Nikon Optiphot microscope and assigned the false colours indicated in the figure legends. Colour prints were obtained using a Sony Color video printer UP5000-P.

Antibodies

The polyclonal rabbit serum Rb271 (against cyclin B) (Whitfield et al., 1990) was used at a dilution of 1 in 500. The rat monoclonal YL1/2 (Seralabs) directed against tyrosinated tubulin was used at 1 in 10 (hybridoma supernatant) and 1 in 100 (ascites).

RESULTS

The identification of the *thr* gene

The embryonic lethality resulting from *thr* mutations is uncovered by the deficiencies *Df(2R)Pcl7B* and *Df(2R)Pcl11B* but not by *Df(2R)PclW5*, thus placing *thr* on the right arm of chromosome 2 between 54F and 55A1 (Fig. 1A). We examined *thr* alleles generated by J. P. Gergen using P-M hybrid dysgenesis to determine whether a P element transposon was inserted at this chromosomal site. The P element probe p 25.1 (O'Hare and Rubin, 1983) hybridized in situ to an element located at 54F3-6, which was present in stocks *thr*^{BH9.6} and *thr*^{BH9.8} but not in the wild-type dysgenic revertant *thr*^{BH9.9}. This offered the possibility of cloning the tagged gene. We therefore constructed a library of recombinant bacteriophage using DNA from flies carrying the *thr*^{BH9.8} mutation, maintained over the *CyO* balancer chromosome, which we screened using both a P element probe and a probe prepared from DNA microdissected from band 54F of wild-type *D. simulans* salivary gland chromosomes and amplified by PCR (see Materials and Methods). DNA from one such double-pos-

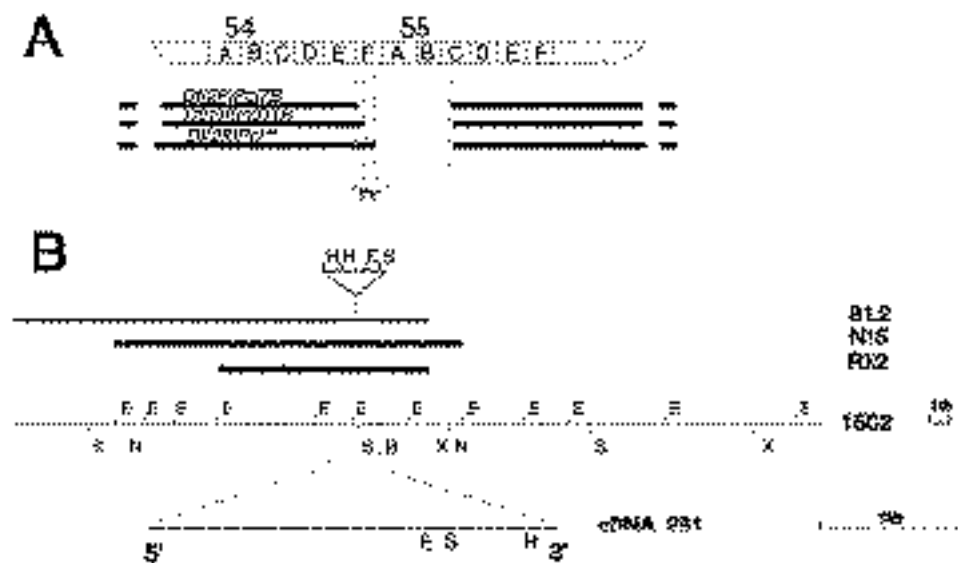


Fig. 1. Cytogenetic and molecular maps of *thr*. (A) Diagrammatic representation of salivary gland divisions 54 and 55 to show regions uncovered by three chromosomal deficiencies. *thr* mutations are uncovered by the deficiencies *Df(2R)Pcl7B* and *Df(2R)Pcl11B* but not by *Df(2R)PclW5*. (B) Map of the *thr* locus. The extent of lambda clone 81.2, bearing the P element from chromosomal region 54F of the *thr*^{BH9.8} chromosome is shown above the restriction map cosmid 15C2 containing the corresponding wild-type DNA. N15 and RX2 are restriction fragments of 15C2 that restore *thr*⁺ function when reintroduced to flies by germline

transformation. The embryonic cDNA 2B1 is shown below on an enlarged scale, the broken lines indicating its relationship to the equivalent region of genomic DNA. E, *Eco*RI; B, *Bam*HI; H, *Hind*III (only sites for P element and cDNA shown); N, *Not*I; S, *Sal*I; X, *Xba*I.

-847 ttaagagaagagctagccatatgaggctattttttattaatctcgat
 -800 atcaataagcaaaaaattgtatcggttatcggaatgagcatggaagtttagtattttccacaagtatttgtgttcagatatgaaaactagaaatctcga
 -700 tgttatcggttaaaggattttccagcttttagcatggacggtcacactggatctcaaaaatctggcgcaaaagcaacaaaaaaggaaagcgctcgagttaaact
 -600 agttaaattgtgccaatccctttgaaaaaatgtctactgatatagccaccagctgaaggcgagccgctccgatgtggaaaaatgtccgcaaaacgggtgg
 -500 aagccaaattccgggagttgtctggcgatggactacctctgagatacgaagtgaatgtattgcgccatatttgccctgccttaaaaggataatctgcacc
 -400 agaactcggaatctttactgcgacatcatgggcataatgctgccacgcgtagttccctgcgaggagaagccaagcttatgggaagcgcaacttgtccagcc
 -300 tgcggtacatacatcatggcttgtttcatcaggtaggagtcgtataagaatcttgcgaatcttttaaccaattotcccgttcacagcgatccattgaa
 -200 gcctgtcagaagctttacaacctcatccgacaacaaccctgccgtctcgaagaggaatcagactacaaaatatacttgacatacatttgaccaccttc
 -100 aatgggtttccatgtgctgctgcagaagcaaaaactccccttggaagctacaagtcaattgtgttatgctttggaatcattggggagatctattcgagcc

1 ATG ACG CAA AGG CAA ATA AGT CTA TGC GCA ACG CTT CTA GTT CAA CTA AAC GAG AGT TTA TTT GGC AAA AGA AGT
 1 M T Q R Q I S L C A T L V Q L N E S L F G K R S S

76 AGA TCA TTC TTC AAG TCC CTA AGC TTC CTG CCG TCG GAG AGT CTT GCC AAA ATG TTC AAC CCG CTG CTT ATG CTC
 26 R S F F K S L S F L P S E S L A K M F N P L L M L

151 CTG GCC AGC AGC ACC AGT TCG AAC CTG GCC AAC TTA TTC CCT GAG TGC CTG AGT CTC ACG TTG GCT CTC GTA CAA
 51 L A S S T S S N L A N L F P E C L S L T L A L V Q

226 ATC GAC ATG TTC AGT CCA CAG TCC AAT CAA CAG ATG TCA TTG CAG CTG CTG CGC ATG AGC AAG GAA CTA TTT CGC
 76 I D M F S P Q S N Q Q M S L Q L L R M S K E L F R

301 CAG GAA TCA AAC CTG TGC TAT GCT CTA CAG CTG ATG TAC TAC TAT ATC AAG TTA ATC TTT GTT CGG GAA CCA ACA
 101 Q E S N L C Y A L Q L M Y Y Y I K L I F V R E P T

376 GGC GAC TTT AAG CGC ACC TAC ATT GAC TTG TCT AGC AAG TTT CAG CAC TTC TTC GAG CAC AAA GTA GCC TCG CAT
 126 G D F K R T Y I D L S S K F Q H F F E H K V A S H

451 GCC AAA GAA CAG TGG CTA GCG GAT TTT CTG GTG GCC ATT CAA TTG CTA CAG GTG CTC ATC CAT CAA AGT AAC AGT
 151 A K E Q W L A D F L V A I Q L L Q V L I H Q S N S

526 AAG TTG CAG AGT CCT TTT CAG ATA TTT TGG CAG CAG TTT GAC GGA GAG AGC AGT CCC GAG ATC TAC ACA GCG CAC
 176 K L Q S P F Q I F W Q Q F D G E S S P E I Y T A H

601 TTT CAG TTG CTT CAG ACG TGT GCT AGC TTG GCG GTT AAT ATT ACG AGG AGT CCT TTG GGC TGC AGT TGC TCC CAC
 201 F Q L L Q T C A S L A V N I T R S P L G C S C S H

676 GAG GCA TGC AAG AGC GTG CGA CGG CAC TGC ATA TTG GCG TAT GGA TTG TGC GCA TTA GAT GCG TAT ATT AAT TGG
 226 E A C K S V R R H C I L A Y G L C A L D A Y I N W

751 AAA CCG GCG GCG GAG CAG AGA GCA AAT GTG gtgggtatttttatctaattttgtggaattttaatgctacatttcaataactattgcag
 251 K P A A E Q R A N V

839 AGC CCT CAC AAG CCC TTG CTG GGA GTA GTC AAA TAC TCA ATG GAT GTG GCT AAG ACC ATG AAG TGC TTG GGT CCC
 261 S P H K P L L G V V K Y S M D V A K T M K C L G P

914 ACC AGT GTG GAG ATC ATT AAG CTA GTG GCG CAG CTG ACA TAC GTG GCT GAT CAG GTC ACC TGT CCG GAG CAA ATG
 286 T S V E I I K L V R Q L T Y V A D Q V T C P E Q M

989 TCC GTG CTG CTG CCA CTT TTG GAG CCA CTG CAG AAG CTG CGA CCT TTG GTT GCC GAC CAG GAT ATG AGC AGT TTA
 311 S V L L P L L E P L Q K L R P L V A D Q D M S S L

1064 CTC CGA CGC CTC TTT AAG GCC AGC TCC CAT TGC GGC GAT TCC AAT ATA GCT TGT CGA ATT CAA GCT AGT TAT TTG
 336 L R R L F K A S S H C G D S N I A C R I Q A S Y L

1139 GCC TCG ATT ACG AAT CCG GCA CGA TTA AGA TCA CAG GTC TGT TTG TAC TAT CAC AAT CTG GGA AAA AAG GGC ACC
 361 A S I T N P A R L R S Q V C L Y Y H N L G K K G T

1214 GAG ATC AAA AGG TGT GTC TAC GAG TGG CAC GAG TCC ACG CCA CTA CCT TTT CCT CTC ACT CCG GAC CAG AAG AAA
 386 E I K R C V Y E W H E S T P L P F P L T P D Q K K

1289 CAG CTG TAT GAT ACC GAT TTC TTT GCC TTA CTA CAC TAT TTG AGG AGT CCT TCT ACG GCT CAT ATG GAA TCA CTA
 411 Q L Y D T D F F A L L H Y L R S P S T A H M E S L

1364 ATT CGT TGC CGA ACG AGT GAC TAT CAT CTG GTA CTC TTG GCC AGA CAA ATG CGA AAC GAT GAC TCG*ATT TCG AAG
 436 I R C R T S D Y H L V L L A R Q M R N D D S I S K

1439 AAA TGC ATA GAG GTT CAT GAT AAG CTA AGG CAA CAA CGT TCG CTC AGT CGA ATG GAT AAC TTG TGC CTG GGC CAC
 461 K C I E V H D K L R Q Q R S L S R M D N L C L G H

1514 GCA AGT GTG GGA CTA CTA CTG GAC GCA CTG GAG GCT CAA AAA ACC AAA GTT TCC ACC AAG GAG ATA ACG GAA AAT
 486 A S V G L L L D A L E A Q K T K V S T K E I T E N

1589 ATG TTC GAG GAG CTG CTA CTC AGC AAG AAT TTA TGG CAG ATG AAC ATA CAA AGG GAG CAG CGA TTG GTC AAC TAT
 511 M F E E L L L S K N L W Q M N I Q R E Q R L V N Y

1664 GCT AGT GAA GCC ATC TCG GCC TTC AGC AAC TTC TTC GAT CGA GCA GAT CAA GAG CCA TTG AGC GCA AAT GAA ACG
 536 A S E A I S A F S N F F D R A D Q E P L S A N E T

1739 TCT ATT GAT TGG GAG GCA TTG ATT GAC GAT GCC ATC GCT ACT GCC AAT GCA CTT TCA AGT ATG GGG TAT CAG TCA
 561 S I D W E A L I D D A I A T A N A L S S M G Y Q S

1814 GAA GAG GAT GAT GCC TGG CTG TTG CTT CTG AGG ATG GGT CGC TTG CTG GAA GAT CGT TTT ACC TAT CTG CGT GCC
 586 E E D D A W L L L L R M G R L L E D R F T Y L R A

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1889 CTA AAT CAT TTT CTG TCA CAG AAT GAG GTT AGT TCT CGT TTA AAT CTG AAA CTC GGC GAG GAA GTG GAA GTA GCA
611 L N H F L S Q N E V S S R L N L K L G E E V E V A

1964 GAG GAA TTG CTG GAT GAT TTG TGG CCT CAA TTG AAA AAT GGC AAA TTC TTC AAG CGT CAG CAA ACT ACG GTA ATG
636 E E L L D D L W P Q L K N G K F F K R Q Q T T V M

2039 CTC TGT TTT TGT CAC CTC GCC AGT TAC TAT GCC AGA ATG GAA TGC TAT AGT CAT GCC CAG TTG CTT CTA TTG CAT
661 L C F C H L A S Y Y A R M E C Y S H A Q L L L L L H

2114 GTG GAA CAA CTT CGC GAA GAG TTT CCT GAG AGA CAA GGA AAA AGT GAT ATT GTA TTG CTT ACA CTG CAA ACG GTG
686 V E Q L R E E F P E R Q G K S D I V L L T L Q T V

2189 CGC TTT CGA ATT GGG TAT CAG CAA AGG AAG CCA ACG AAT TGC AGG CTG CCG ACT CCT CTG CGT CAA TTG GAC ATT
711 R F R I G Y Q Q R K P T N C R L P T P L R Q L D I

2264 CTT CTG GAC AAT GTG CGA AGT TTT TGC AAT CTA TCC AGT TTA GAT GGC GGC TCA TTG CAG CTG CTT CTT TCG ACT
736 L L D N V R S F C N L S S L D G G S L Q L L L S T

2339 CTT GTC AGG GAA AGC ACC GAG TGC TCT GCG AAC AGA TTA AGC GAA AGA CTG TCC TTC TCC AAC ATT GCA CTA CAT
761 L V R E S T E C S A N R L S E R L S F S N I A L H

2414 TTG GTA CTC CAA TCT GGC TTG GCT TTA AGA GCC ATA GAG GTA TTC CTT GCA TGG TTA TGG ACC AAT TTG CAA ATG
786 L V L Q S G L A L R A I E V F L A W L W T N L Q M

2489 GAA AGT TTC GAC AAG GCG CAA TCG AAG TTG AGA CTC ATC GAG CAT TGT TTA GGT ATA AAA CAG CTG AAT CCA ACG
811 E S F D K A Q S K L R L I E H C L G I K Q L N P T

2564 AGT AGG CCG GAA AAG GAA GCG ATT AAG GAT GTA GCA ATT AGT GAT CTG GCT AGT AAT ATG CAT CTC CTC CAA TTG
836 S R P E K E A I K D V A I S D L A S N M H L L Q L

2639 GTG GAG CCG ATC AGG AAG CAG CAG CTA TTA AAT ATG GCC TCG CCG AAT CTG CTC AAA ATG CGA CCA CAT AGC CCA
861 V E P I R K Q Q L L N M A S P N L L K M R P H S P

2714 AAT CCC CAA CTG GAC TTG GAT CGC TAC ATA ACA CTC GAT GTG GCG CCA GCG AAT CTT CGA GAG AAT TCC CAG CTG
886 N P Q L D L D R Y I T L D V A P A N L R E N S Q L

2789 CAA TGC TTG TAC TTT GTA ACG GGC TGT CTA CAT GCA CGT CTC CGC TTT CTG CAG AGA AAC AGC GAA CAA TTG GAG
911 Q C L Y F V T G C L H A R L R F L Q R N S E Q L E

2864 GAG TTC TAT GGA AGA GCG CAT AAC TGG ATG CAG GAA AAA CCT CCG ATG AGT AGC GCT TTG TAT CCC ATG TTG CAT
936 E F Y G R A H N W M Q E K P P M S S A L Y P M L H

2939 GCC CAG CAG CTG TAT CAT CTC AAC TAT CTT CGC TTT GCG CGA AAG CAT GTA GAG GCT ATA TCA ACG GCT CAA TTG
961 A Q Q L Y H L N Y L R F A R K H V E A I S T A Q L

3014 GGC CTT AAA ATG CGA TCA CGC GCG GTC GAC ATT AAT TTC GAG TAC AAT TTT TTG GCT CAG CTA AAG ACG GCT CAG
986 G L K M R S R A V D I N F E Y N F L A Q L K T A Q

3089 TTA GAA CTC AAA CCC GTG GGC CAG GAT AAG CCA CAG GTC AAA ATC CTT CGA CGT GCT TTG GTA TTC AAT CAC TCA
1011 L E L K P V G Q D K P Q V K I L R R A L V F N H S

3164 CCC GAA GAT AAG AAA CGA ACC GCA ACA GGA TCG GTT TCA GCA GTC AAG AAT ACG GCG TCT AAA GTT AAA CAG TCG
1036 P E D K K R T A T G S V S A V K N T A S K V K Q S

3239 GCC AAA AAG GCA CCT CGA TTC AGA ATT TAC GAG GAG CTG GAA CTA CGA CCA CCA AGT GCT ACC AGT TGC AGT AGC
1061 A K K A P R F R I Y E E L E L R P P S A T S C S S

3314 AGC GGT GGC AGC GGA ACA GAG AAT ACA CCG CCT TCG GAT CAC GTG GAT CTA AAT GCC TGT CAA GCG ATC GAG ATA
1086 S G G S G T E N T P P S D H V D L N A C Q A I E I

3389 AGC GAC GAT GAC GAT TCA CCT TTG GTG TCC ACA AAG AAG ACA CAA CCA AAA AGT AGG GAG AAG GCA AAA CCC AAG
1111 S D D D D S P L V S T K K T Q P K S R E K A K P K

3464 GCC ACA TCC AAA GCT TGT AAA GTC CTA ACA TTG GAT AAT AGC TTG GAA ATA GTA GAA ACG CCG ACA ATA ACT ACA
1136 A T S K A C K V L T L D N S L E I V E T P T I T T

3539 AGT ACA CGG AGC ACC AGA GCC AGG CTG CGC AAC CAG TAG agacaccaaagacagcgactctttcatccaagcggaacgaggcgccag
1161 S T R S T R A R L R N Q

3625 gtttttgaagcacaggtcccgaaacggagtccattagcacacgcacacggcatcggcactgattagatagtagtctccttagttcgattatttctta
3725 tcgaaaatgtcttggtattttaatatatatattgtgtgt

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Fig. 2. Sequence of the *three rows* transcription unit. Nucleotide and predicted amino acid sequence of *thr*. The first bold-type methionine residue following the in-frame stop (nucleotides –54 to –52) shows a 5/14 match to the published translation initiation consensus (Cavener and Ray, 1991). The second bold-type methionine shows a 8/14 match, maintaining all five of the most conserved (capitalised) residues in the consensus cacaacAaaATGgc. Upper case letters represent residues conserved in more than 50% of genes studied (Cavener and Ray, 1991) and lower case letters indicate the most frequent base at a position where no base occurs more than 50% of the time. * indicates a position (nucleotide 1429) where we read a C residue in a genomic clone and a G residue in a cDNA clone. A poly(A) tail follows the last indicated nucleotide.

itive recombinant phage identified in this screen, 81.2 (Fig. 1B), carries a segment of *Drosophila* DNA bearing a 2.9 kb P element and was used to screen a cosmid library of wild-type *Drosophila* DNA (Materials and Methods). One of the resulting clones, 15C2, hybridises to 54F3-6 in situ and contains 35 kb of DNA around the site of the P element insertion found in the *thr*^{BH9.8} chromosome (Fig. 1B). A comparison of the restriction maps of the phage 81.2, the

cosmid 15C2 and an embryonic cDNA, 2B1, isolated using probes from the P element insertion site (Materials and Methods) indicated that the P element in *thr*^{BH9.8} is inserted in a *SalI-EcoRI* fragment of approximately 200 bp, which is present in both genomic DNA and the cDNA. It is therefore likely that the P element disrupts the expression of an embryonic transcript.

In order to confirm that the *thr* gene lies at this site, we

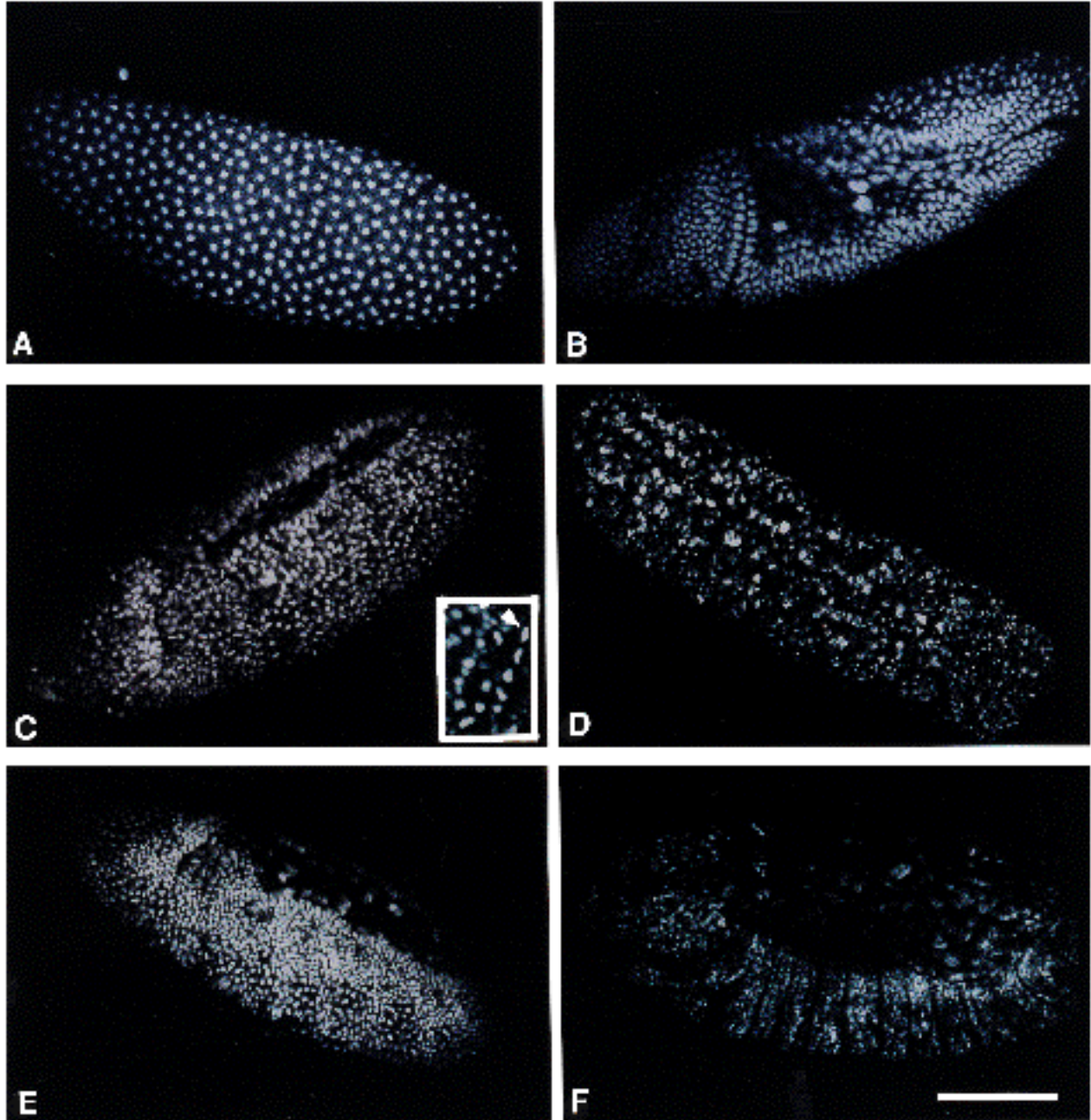


Fig. 3. Development of *thr* (A-D) and wild-type (E,F) embryos. Embryos have been fixed and stained with propidium iodide to reveal DNA as described in Materials and Methods. *thr* embryos in (A-C) are at the following division cycle stages: (A) cycle 12; (B) cycle 14; (C) cycle 15. The embryo in (C) shows mitotic domains that appear larger than usual as they have coalesced. The inset in (C) shows a twofold enlargement of one of these areas. Corresponding metaphase figures are indicated by arrowheads in the main panel and in the inset. No further cell division occurs following cycle 15, and embryos develop the appearance shown in (D) by 7 h AED. The wild-type embryo (approx. 5.5 h AED) in (E) is undertaking mitosis 15. A later embryo (approx. 7 h AED) is shown in (F) following germ-band retraction. Bar, 100 μ m.

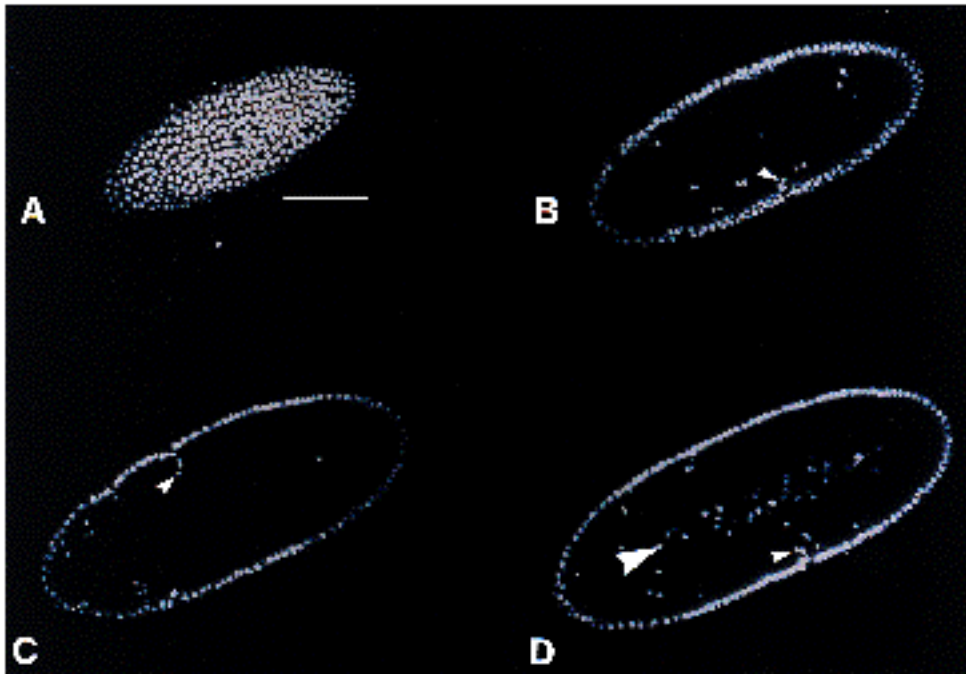


Fig. 4. Optical sections of a syncytial *thr* embryo. The embryo shown has been stained with propidium iodide to reveal DNA. The uppermost section is shown in (A) with sections at 5 μ m intervals in subsequent panels. The large arrowhead in (D) shows the yolk nuclei, which are found in the centre of the embryo. The small arrowheads in (B) and (C) indicate nuclei that have sunk from the cortical region where they are more normally found. Bar, 100 μ m.

carried out P element-mediated germ-line transformation experiments (Spradling and Rubin, 1982) utilising the DNA segments RX2 derived from the cosmid 15C2 (Fig. 1B). The RX2 construct restored viability to *thr*^{1B} and *thr*^{SJB22} homozygotes, indicating that no sequences necessary for wild-type *thr*⁺ function lie outwith this region (Materials and Methods).

Sequence analysis of *thr*

The open reading frame in the DNA sequence of cDNA 2B1 revealed the clone to be incomplete, lacking 5 sequences (see Materials and Methods). We therefore also determined the sequence of genomic DNA encoding the N-terminal region of the *thr* protein. In addition we used a PCR-based approach to obtain the 5' end of the *thr* tran-

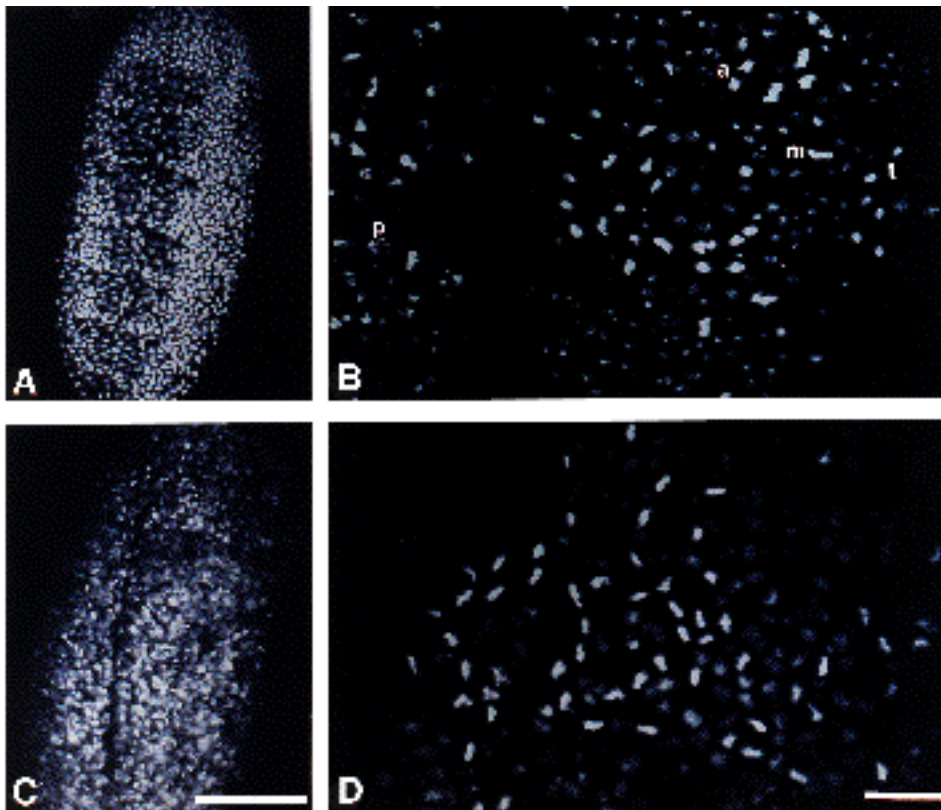


Fig. 5. Wild-type and *thr* embryos in cycle 15. Wild-type (A and B) and *thr* (C and D) embryos are stained with propidium iodide. The wild-type arrangement of mitotic domains can be seen in the low-power view of a wild-type embryo in (A). The higher magnification view in (B) reveals cells at all mitotic stages: prophase (p), metaphase (m), anaphase (a) and telophase (t). The *thr* embryo shows abnormally large domains of mitotic figures (C), which can be seen at higher power to contain mainly metaphase figures (D). Bars, 100 μ m (A and C) and 25 μ m (B and D).

script and have defined the start of the *thr* transcript to be -722 ± 5 nucleotides upstream of the first in-frame start codon. The DNA sequence thus allows us to predict the size of the mRNA as 4432 nucleotides excluding the poly(A) tail. This is in excellent agreement with the results of northern analysis (data not shown). The transcripts have a similar distribution throughout early development to those of other genes essential for cell cycle functions. In situ hybridisation to embryos shows an abundant maternal contribution of *thr* mRNA to the syncytial embryo. These are excluded from newly formed cells during the interphase preceding mitosis 14 and are replaced in subsequent cycles by zygotic transcripts, present at particularly high levels in the developing central nervous system (data not shown).

The sequence of *thr* is presented as Fig. 2. The highlighted M at residue 1 is the first start codon in the reading frame but is not the best fit to the published consensus for translation start sites (Cavener and Ray, 1991). The second methionine (amino acid 43) best fits this consensus and is also a likely candidate for the actual translation start site. In this latter case the gene would encode a polypeptide of 1130 amino acids. The *thr* gene product bears little resemblance to any sequence reported in the databases. The only homology of possible significance detected is with the *nuc2* gene product of *Schizosaccharomyces pombe*. The match over a 26 amino acid sequence is as follows (|, identities; :, conservative matches):

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thr534NYASEAISAFSNFFDRADQEPLSANE559
      :|::: |||||:: ||| | |:::|
nuc2219SYSNSSISAFTKWFDRVDASELPGSE244
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This match occurs within the DNA binding domain of the *nuc2* gene product, a nuclear scaffold-like protein that

appears to be required for chromosome disjunction (Hirano et al., 1988, 1990; Masuda et al., 1990.)

Aberrant nuclear divisions occur at low frequency in syncytial *thr* embryos

In order to search for mitotic abnormalities predicted by the cuticle phenotype, we first examined the organisation of chromatin in embryos lacking zygotic *thr* function (hereafter called *thr* embryos). The recessive *thr* mutation is maintained in a heterozygous stock with the *CyO* balancer chromosome, which is marked with the *E. coli lacZ* gene. This enables homozygous *thr* embryos to be distinguished from their siblings by the lack of histochemically detectable β -galactosidase activity. *thr* embryos at successively later stages of development are shown in Fig. 3A to D. The normal distribution of nuclei in syncytial embryos (Fig. 3A) and newly cellularised embryos (Fig. 3B) suggests that mitosis takes place correctly in cycles 1-14. Mitotic abnormalities become strikingly evident in cellularised embryos during the second cell cycle to follow cellularisation (mitosis 15, Fig. 3C) and can be seen as large areas of cells delayed in mitosis (see below). The mutant embryo in (C) should be compared with the wild-type embryo at a comparable stage in (E). *thr* embryos subsequently develop large areas of cells that contain disorganised chromatin (Fig. 3D). The nuclei of many of these cells are considerably larger than those in wild-type embryos of comparable age (Fig. 3F).

A more careful inspection of *thr* embryos at syncytial blastoderm revealed that a small percentage of nuclei lose their association with the cortex during cycles 11 to 13 and appear to sink into the interior of the embryo. This can be seen in the four optical sections of a propidium iodide-stained embryo presented as Fig. 4. The upper- and lower-

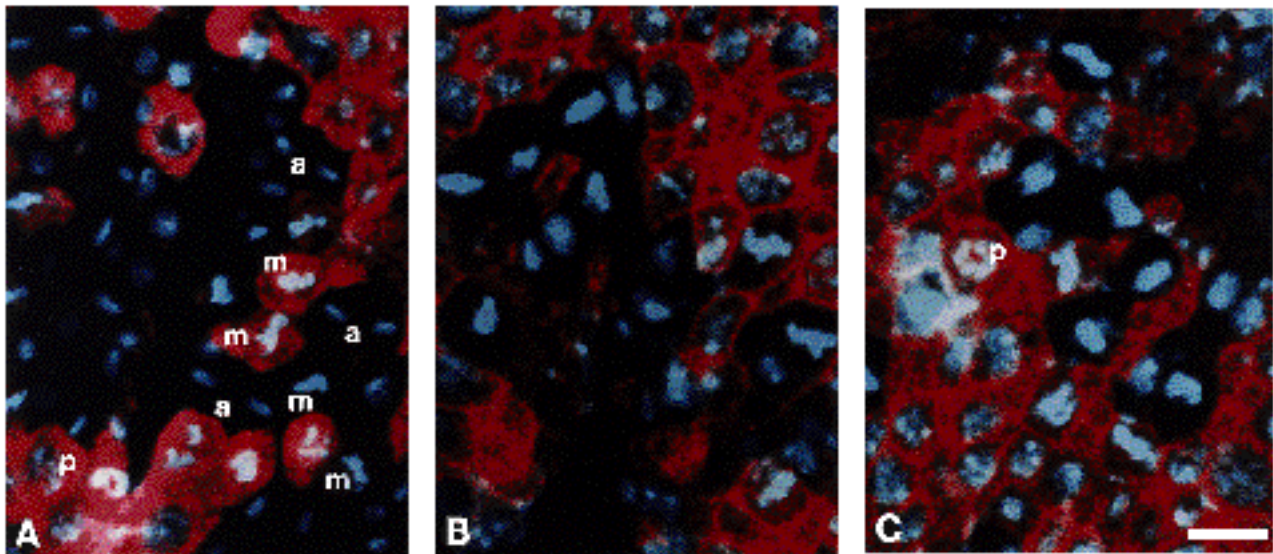


Fig. 6. Cyclin B degradation in mitotic domains of wild-type (A) and *thr* (B,C) embryos. Embryos have been stained with propidium iodide to reveal DNA (blue) and with the anti-cyclin B antibody described by Whitfield et al. (1990) to reveal cyclin B protein (red). In the wild-type embryo (A) cells can be seen at all mitotic stages: prophase (p), metaphase (m) and anaphase (a) are labelled. Cyclin B has been degraded in about half of the cells that have metaphase figures and in all the anaphase cells. In *thr* embryos (B and C) only prophase (p) and metaphase-like figures are visible. In embryos of this stage more than 95% of the metaphase cells lack cyclin B and there are no anaphase figures. Bar, 10 μ m.

most sections are through the layer of nuclei at the cortex of the syncytium (A) and the layer of yolk nuclei in the middle of the embryo (large arrowhead, D), respectively. Nuclei that have sunk below the cortex (small arrowheads, B and C) can be seen in the intermediate focal planes. We did not observe any such sinking nuclei in sibling embryos, which carried a *thr*⁺ allele on the *CyO* chromosome. This phenotype is similar to that previously described as a consequence of abnormal centrosome separation in the maternal effect mutant *daughterless-abo-like* (Sullivan et al., 1990), but considerably less severe. The low frequency with which this phenomenon occurs (less than 1% of nuclei appear to be affected in *thr* embryos) has meant that we have not been able to assess the nature of any mitotic defect that might lead to the phenomenon (see discussion).

Delay in metaphase during cycle 15 of cellularised embryos

We have examined the pattern of cycle 14 mitotic domains by following the behaviour of chromosomes stained using propidium iodide; spindle microtubules revealed by immunostaining; the nuclear antigen that relocalises from the nucleus to the centrosome during mitosis (Whitfield et al., 1988); and A- and B-type mitotic cyclins (data not

shown). We conclude by each of these criteria that the order of entry into mitosis of each domain is as described by Foe (1989). We were unable to detect any mitotic abnormalities. We have also carried out pulse-labelling studies with BrdU that indicate that DNA synthesis occurs at the normal time, immediately following mitosis 14.

A comparison of the cycle 15 mitotic domains in wild-type and *thr* embryos is shown in Fig. 5. Whereas the wild-type domains contain cells at all stages of mitosis (A and B), anaphase figures are not observed in *thr* embryos at this developmental stage, and instead mitotic domains contain predominantly metaphase figures (Fig. 5C and D). The mitotic domains of cycle 15 normally contain fewer cells than those of cycle 14. In *thr* embryos, however, the domains coalesce, giving rise to large patches of cells, all of which are at metaphase. We conclude that entry into mitosis 15 occurs normally but that cells are delayed in their mitotic progression after chromosome congression to the metaphase plate and before the separation of chromatids characteristic of anaphase.

Some aspects of mitosis proceed in the absence of chromatid segregation

In the cells of wild-type embryos, the mitotic cyclins accu-

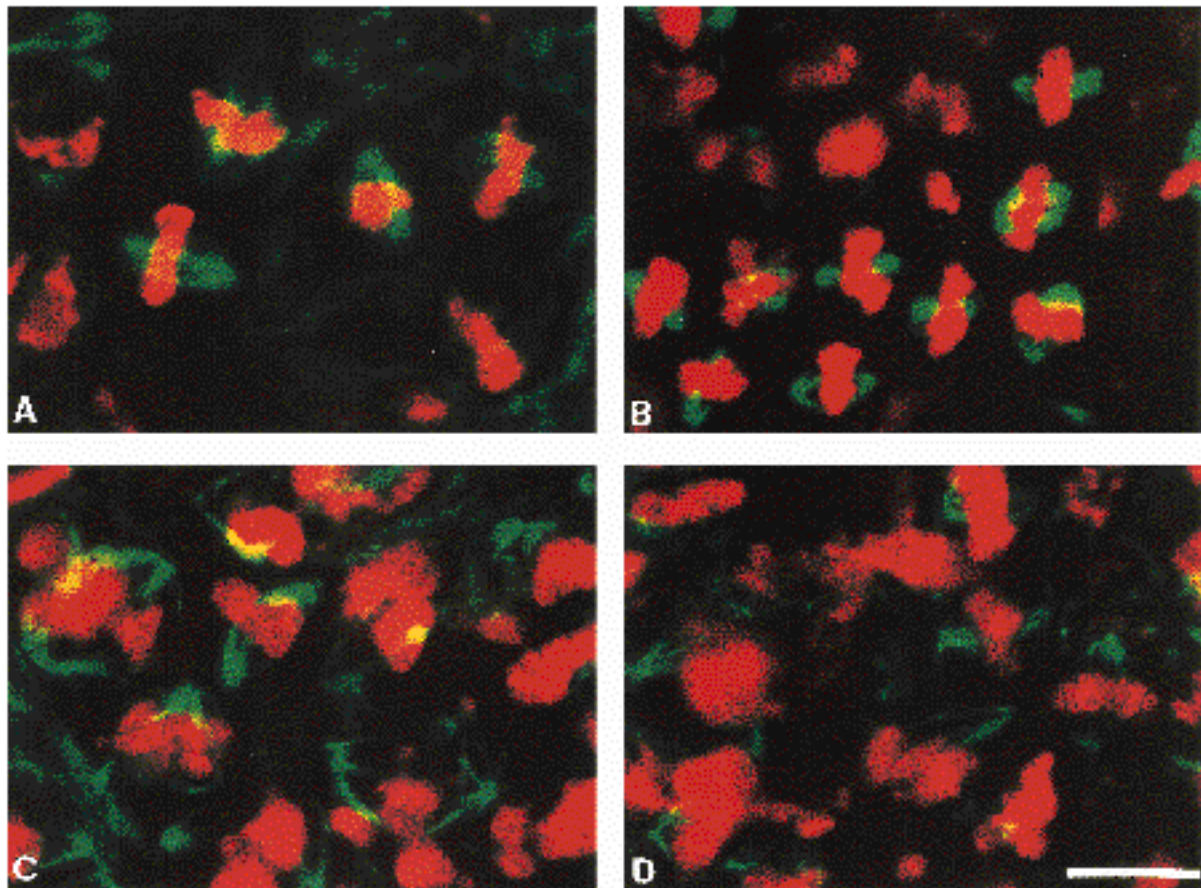


Fig. 7. Bipolar spindle structures in *thr* embryos. Embryos were stained for DNA (red) using propidium iodide, and for tubulin (green) using the YL1/2 monoclonal antibody (Kilmartin et al., 1982; Sera-lab). (A) to (C) show bipolar spindles associated with metaphase configurations of chromosomes. (C) and (D) are from a different embryo at a later stage in which the chromatin is decondensing. No chromatid separation is seen. Bar, 10 μ m.

mulate throughout interphase and into prophase (Lehner and O'Farrell, 1989, 1990; Whitfield et al., 1990). The cyclins are degraded at the metaphase-anaphase transition, cyclin A slightly ahead of cyclin B. Metaphase cells containing cyclin A are rare, whereas cyclin B is still present in about 50% of metaphase cells but has been completely degraded by the time that chromosome segregation occurs at anaphase (see Fig. 6A). In *thr* embryos cyclin B also accumulates in interphase and prophase cells but is not detectable in those cells delayed in metaphase (Fig. 6B and C). Cyclin A is also absent in these delayed cells (data not shown). Thus, it seems that the *thr* mutation delays metaphase in terms of chromatid separation, even though the metaphase-anaphase transition would appear to have taken place by the criterion of cyclin B degradation.

We wished to determine how this abnormal metaphase-anaphase transition might be resolved in *thr* cells and therefore carried out immunostaining to examine the organisation of the spindle microtubules (Fig. 7). We were able to detect bipolar mitotic spindles associated with chromatin in two different states. In the two mitotic domains shown in Fig. 7A and B, most cells have a metaphase configuration of chromosomes (red) associated with a bipolar mitotic spindle (green). This is the most common arrangement of *thr* spindles, and corresponds to the metaphase delays we have already described. Fig. 7C and D shows domains, from *thr* embryos, that appear to be at a later stage in these abnormal mitoses. The chromatin has remained at the position of the metaphase plate and is becoming decondensed as if at telophase.

DISCUSSION

A severe mitotic phenotype first becomes apparent in the *thr* embryos during cycle 15. This suggests that the maternal provision of the *thr* gene product is sufficient to permit the embryo to develop more or less normally to this stage. This is reflected by the abundant supply of *thr* RNA detectable by in situ hybridisation in syncytial embryos (data not shown). Nevertheless, the finding of nuclei that from their position appear to be sinking from the cortex into the interior of the syncytial blastoderm embryo suggests that some defective mitoses might be occurring at this stage. We only detect this phenotype in embryos homozygous for *thr*, and not in their siblings that will have received an equivalent, most likely reduced, contribution of the *thr*⁺ gene product from their heterozygous mothers. It seems likely therefore that the levels of the *thr*⁺ gene product in the syncytium of these embryos are near a critical threshold and must be supplemented by a zygotic contribution in order for all nuclei to divide normally. Such a contribution would come from expression of *thr*⁺ in the unaffected embryos during syncytial blastoderm when low levels of expression are known to commence from several other genes. We have been unable to observe the nature of the mitotic defect at this developmental stage, and suggest that if the mutation results in a metaphase delay, as occurs in the later divisions, it would be difficult to detect in less than 1% of nuclei in a fixed preparation. The consequences of the aberrant division are more readily seen, however, in

that the affected nuclei become abnormally located, and result in a small disruption of the monolayer of nuclei at the cortex around which unaffected nuclei continue to divide, thus leaving a 'footprint' that points to previously abnormal division. We are currently undertaking real time observations of mitoses in syncytial *thr* embryos to attempt to resolve this problem. Our failure to detect any aberrant mitoses in cycle 14 does not mean they do not occur. The asynchrony of divisions in mitotic domains accentuates the difficulty in detecting such events if they take place at low frequency.

The dramatic increase in abnormal mitoses during cycle 15 suggests that the maternal provision of *thr*⁺ protein falls below a critical threshold at this time. In wild-type embryos, the majority of maternal *thr* transcripts are degraded at cellularisation, in common with many other mRNAs for cell cycle proteins (see Whitfield et al., 1989, for example). Although we do not as yet have antibodies to the *thr*⁺ protein, and so cannot study its levels, we suspect that the maternal *thr* protein must persist beyond cellularisation, since the initial levels of zygotic transcription of the gene appear low. The zygotic transcripts subsequently increase considerably in the developing central nervous system (data not shown), a tissue that retains its mitotic potential throughout all of larval and pupal development.

Although all cells within a cycle 15 mitotic domain appear to be equally affected by *thr* mutations, the phenotype is not a cell cycle arrest, but rather a metaphase-like delay, followed by an apparent continuation of certain aspects of the cell cycle in the absence of chromatid segregation. This is perhaps surprising, since in the yeasts and in cultured mammalian cells, several dependency relationships have been described whereby the execution of a particular cell cycle event does not occur unless a previous step has been correctly completed (Hartwell and Weinert, 1989). Thus blocking the yeast cell cycle in S phase prevents subsequent mitosis (Slater, 1973), and disruption of microtubules leads to a mitotic arrest that prevents the next G₁ phase from taking place (e.g. see Huffaker et al., 1988). The existence of feedback mechanisms that regulate passage through these 'checkpoints' has been suggested by the discovery of mutants that fail to pause in response to DNA damage (Weinert and Hartwell, 1990), or fail to arrest in mitosis in response to loss of microtubule function (Li and Murray, 1991; Hoyt et al., 1991). The checkpoint monitoring microtubule integrity would seem to be related to the need to assemble the mitotic apparatus correctly before proceeding with chromosome segregation. The drug-induced disruption of microtubules leads to a metaphase-like arrest in cells from a variety of organisms. Cyclin A within such arrested cells has undergone degradation, whereas cyclin B is maintained at high levels (Whitfield et al., 1989; Minshall et al., 1989). We assume that this arrest is at the checkpoint where correct assembly of the mitotic spindle is assessed. The delay produced by *thr* mutations, however, appears to be at a later stage in mitosis. Cells accumulate in metaphase with their chromosomes aligned on a normal-looking spindle with both cyclins A and B having been degraded. It seems that the *thr* mutation prevents sister chromatid segregation, but that the resulting cell cycle delay occurs after the checkpoint for microtubule integrity. As the

regulation of the segregation of chromatids into daughter cells at anaphase is crucial in cell division, the failure of cells to arrest at the known microtubule integrity checkpoint, before cyclin B degradation, in *thr* embryos was unexpected. This finding may therefore define a new checkpoint regulating chromatid segregation which is absent in *thr*. Alternatively, *thr*⁺ may have a purely mechanical function in mediating chromatid separation, and checkpoints, that detect the aberrant events of cycle 15, may only operate in the subsequent mitosis.

Mitosis normally consists of a linear progression of events. In the absence of *thr*⁺ function, however, cells appear to accomplish only a subset of events in this linear progression: although cyclin B is degraded as normally happens at the metaphase-anaphase transition, the chromosomes maintain an appearance characteristic of metaphase and do not undergo any anaphase segregation. Telophase does occur in that the chromosomes subsequently decondense, and this is later followed by DNA replication. The linear execution of parts of the mitotic process in the absence of others in *thr* cells suggests the existence of parallel mitotic pathways that are normally coupled together. It is possible that *thr* functions as part of such a coupling mechanism, or that it is required to act in one of the pathways at the time at which coupling is achieved.

Such coupling processes may be related to the mitotic checkpoints, which appear to be introduced sequentially into the *Drosophila* cell cycle as development proceeds. Thus, in syncytial embryos (cycles 1-13), there appears to be no checkpoint coupling mitosis to the completion of S-phase (Raff and Glover, 1988). This dependence is only introduced following cellularisation in cycle 14, so that progression through cycles 14, 15 and 16 appears to be regulated primarily at the G₂-M transition by *string*, a *Drosophila* homologue of the fission yeast *cdc25* gene (Edgar and O'Farrell, 1989, 1990). A G₁ phase is not introduced until after cycle 16. Regulation in cell cycle progression at G₁ is known to occur in the central nervous system during larval development (Selleck et al., 1992). As *thr* is highly expressed in neural lineages, the cells of which go on to divide extensively after mitosis 16, it will be of interest to examine the consequences of the *thr* mutations in such tissues at these and later developmental stages. Such an analysis would involve making mutant clones of *thr* cells, for example by mitotic recombination, in a heterozygous animal.

The finding of a small region of similarity with the fission yeast gene *nuc2* is intriguing, as mutations in this gene block chromosome segregation and lead to metaphase arrest in fission yeast. However, we do not see any repeating amino acid domains in the *thr* protein, a characteristic structural feature of *nuc2*. The *nuc2* protein is present in the scaffold-like fraction of *S. pombe* cell extracts, and also contains a DNA binding domain. We are currently raising antibodies against the *thr* protein produced in *E. coli* in the hope that such reagents will help us study its sub-cellular localisation in *Drosophila* cells, and its interaction with other molecules.

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slein-Volhard for pointing out to D.M.G. in 1984 that *thr* was likely to be a mitotic mutant. We also thank Alfonso Martinez-Arias for drawing our attention to the dysgenic *thr* alleles. We are especially grateful to Sarah Bray for providing the marked *CyO ftz-lacZ* balancer and for the previously unpublished *thr^{SJB22}* allele. In the course of this work we became aware that the laboratories of Robert Saint and Christian Lehner were carrying out similar experiments. They have been extremely helpful in generously exchanging data prior to publication. Flies were obtained from the Tübingen and Bloomington stock centers. Finally we thank our colleagues in the lab. Their robust criticism is always enlightening. The work was supported by the Cancer Research Campaign and the Wellcome Trust. A.V.P. holds a Wellcome Trust studentship.

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